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Detergent-Inhibited, Heat-Labile Nucleoside Triphosphatase in Cores of Avian Myeloblastosis Virus

KAJ FRANK JENSEN†

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

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Endogenous DNA synthesis was studied in isolated core particles of avian myeloblastosis virus. It was found that cores contained an enzymatic activity which rapidly converted the added nucleoside triphosphates to diphosphates (but not further) at 0°C, thus inhibiting DNA synthesis. This triphosphatase probably originates from the viral membranes. In the cores the enzyme is completely inactivated by low concentrations (0.02%) of Nonidet P-40. Also, the enzyme is very thermolabile and denatures rapidly at 38°C.

Virions of RNA tumor viruses are roughly spherical particles composed of a core component (a ribonucleoprotein particle) surrounded by a unit membrane (18). The core component can be separated from the membranes by isopycnic banding in sucrose gradients after disruption of virions with nonionic detergents (5, 15). After this treatment the endogenous DNA-synthesizing capacity is found in association with the core component (5, 15). The major polypeptide components of virions and cores of avian myeloblastosis virus (AMV) have been extensively characterized (15, 16).

RNA tumor virions contain, associated with their membranes, many enzymatic activities that interfere with DNA synthesis; most or all of these enzymes are of cellular origin (12, 18).

The DNA synthesis of RNA tumor viruses is usually studied in a "crude" system after disruption of virions with nonionic detergents (5, 6, 9, 13) or in a system composed of phenol-extracted viral RNA and purified viral DNA polymerase (1). The isolated cores were expected to be a purer system than the crude disrupted virions. Simultaneously, the cores might contain some protein components that are absent from the reconstituted system but are needed for efficient DNA synthesis, as found for DNA synthesis in bacteria (20). This communication describes the presence of a nucleoside triphosphatase in cores of AMV. In the absence of detergent this enzyme extensively damages the deoxynucleoside triphosphates even at 0°C. However, in cores the enzyme is rendered highly thermolabile and is completely inhibited by low levels of Nonidet P-40 (NP-40). When the phosphatase is inhibited, the cores carry out a very efficient DNA synthesis that proceeds for hours at 38°C.

MATERIALS AND METHODS

Materials. Viremic chicken blood plasma containing 5×10^{11} to 9×10^{11} AMV particles per ml was a gift from J. W. Beard. Virions were isolated from the plasma as described (10); pellets were stored frozen at −70°C.

^{3}H-labeled 28S, 18S, and 4S cellular RNAs were gifts from A. M. Q. King (10). Poly(rA)$_n$ (size about 4S) was obtained from Pabst Chemical Co. Oligo(dT)$_{10}$ was a gift from J. Burd (4).

[*methyl*-^{3}H]TTP (specific activity 19.2 Ci/mol) and α-[^{32}P]ATP were from New England Nuclear. Unlabeled nucleotides were from Pabst.

Nonidet P-40 was obtained from Shell. Other chemicals were from Sigma, Calbiochem, Malinckrodt, Baker, or Schwarz/Mann.

Before use, all glassware was baked at 350°F (ca. 177°C) overnight. Buffers were made up in doubly distilled water and autoclaved when possible. Polyallomer tubes were autoclaved; cellulose nitrate tubes and gradient makers were washed in 1 M sodium hydroxide followed by doubly distilled water.

Preparation of AMV cores. Pellets of AMV virions were suspended in NET buffer (100 mM sodium chloride, 1 mM EDTA, 10 mM Tris-hydrochloride, pH 7.2) to an optical density at 540 nm of about 1.0 using a Potter homogenizer. Dithiothreitol was added to a final concentration of 0.2%. Then 0.1× volume of 2.5% (vol/vol) NP-40 in NET was added, followed by gentle mixing. Immediately, a strong decrease of turbidity was seen. The suspension was layered over a 30 to 70% linear sucrose gradient in 50 mM Tris-hydrochloride (pH 8.1)-1 mM EDTA-0.2% dithiothreitol. The 30% sucrose stock was made up in water; in the 70% sucrose, 90% of the water was D_2O. A 2-ml sample of suspension was layered over a 3-ml gradient in a cellulose nitrate tube (0.5 by 2 inches; ca. 12.7 by 50.8 mm) and centrifuged for 2.5 h at 45,000 rpm in an SW50.1 rotor. All operations were carried out at 0 to

† Permanent address: Enzyme Division, University Institute of Biological Chemistry B, Copenhagen K, Denmark.

4°C (5, 15). The cores banded at a density of 1.26 to 1.29 g/ml; they were tapped through a hole in the bottom of the tube. By dodecyl sulfate gel electrophoresis (19), the cores were found to have a polypeptide composition typical for AMV cores (16). The 35S RNA subunits of the cores were found to be as intact as the 35S RNAs of the virions used for preparation of the cores (i.e., approximately 30% were full length). This was determined by parallel phenol extraction (10) of RNAs from virions and cores, ^{125}I iodination of the RNAs (7, 8, 10), and analysis of the iodinated, heat-denatured RNAs by electrophoresis in 2.2% polyacrylamide gels (10). The cores were always used on the day of preparation and never dialyzed.

DNA synthesis in cores. The transcription reactions were carried out in 50 mM Tris-hydrochloride buffer (pH 8.1) containing 60 mM sodium chloride, 7 mM magnesium acetate, 1 mM EDTA, 0.2% dithiothreitol (13), and frequently 0.02% NP-40 and/or 1.0 mM ATP at 38°C. The concentration of [^{3}H]TTP (specific activity, 2.2 Ci/mol; 1,180 cpm per pmol) was 0.011 mM; the concentration of the three unlabeled triphosphates was 0.10 mM. The concentration of cores in the reactions ranged from 5×10^{12} to 2×10^{13} cores per ml (details given below). This concentration is expressed as virion equivalents assuming a 100% yield of the core preparation. The amount of virions used was calculated from their concentration in the chicken blood plasma, as given by J. Beard and based on the adenosine triphosphatase assay (2). The reactions were assembled on ice; the cores were added last. Because cores were used directly from the sucrose gradients, the reactions also contained about 10% sucrose and about equivalent amounts of D_2O. DNA synthesis was monitored by spotting 25-μl samples of the reactions on Whatman 3 filters, which were then washed four times in cold 5% trichloroacetic acid (10 min each), two times in 95% ethanol, and finally once in ether. Radioactivity retained on the filters was determined by liquid scintillation counting.

Determination of DNA polymerase activity in reactions primed with poly(rA)$_n$·oligo(dT)$_{10}$ was done as described (3).

Degradation of [^{3}H]TTP. Reactions were composed as described above. At times indicated after mixing, 25-μl samples were pipetted into 25 μl of cold 2.5 M perchloric acid containing 6 mM dTTP (containing some dTDP) and 3 mM dTMP as chromatographic markers. After 7 min the samples were neutralized by addition of 62.5 μl of 1 M potassium hydroxide. The potassium perchlorate was allowed to settle, and 20 μl of supernatant was spotted on Whatman 1 chromatographic sheets (23 by 57 cm). dTTP, dTDP, and dTMP were separated by descending chromatography in isobutyric acid-water-concentrated ammonium hydroxide (66:33:1). The nucleotide markers were located under UV light. The spots were cut out, and the radioactivity was determined by liquid scintillation counting.

RESULTS

Kinetics of transcription in cores; the effect of NP-40 and ATP. Since the cores were prepared by disrupting the virions with 0.25% NP-40 and subsequently sedimenting (and banding) the cores out of the detergent solution, we reasoned that no permeability barrier to nucleotides would be present in the cores. Hence, we did not add detergent to the core suspension when measuring DNA synthesis. However, the cores were found to carry out a variable and inefficient DNA synthesis under these conditions. Addition of a small amount (0.02%) of NP-40 strongly stimulated (3- to 100-fold; Table 1) DNA synthesis. This stimulation was seen only when the detergent was added prior to or simultaneously with the deoxynucleoside triphosphates (Fig. 1A). Addition of the detergent after a short time at 38°C resulted in no stimulation of DNA synthesis (Fig. 1A). This latter result indicated that the cause of the low rate of DNA synthesis was not a permeability barrier in cores which was overcome by the detergent. It was also found that ATP stimulated DNA synthesis in the cores. In contrast to NP-40, the stimulation by ATP was seen also when this compound was added to the reactions after incubation for some time at 38°C (Fig. 1B). This result indicated that the low rate of DNA synthesis in the absence of NP-40 and ATP was due to depletion of deoxynucleoside triphosphates. However, the

TABLE 1. *Effect of preincubation at 0°C and NP-40 treatment of cores incubated with deoxynucleoside triphosphates*[a]

Preincubation (min)	Endogenous polymerase activity (production)[b]		
	A	B	C
120	0.4 (2)	72 (131)	0.8 (3)
60	0.7 (2)	104 (158)	1.3 (4)
30	1.3 (4)	118 (188)	4 (17)
0	37 (50)	137 (215)	126 (215)

[a] Cores were diluted in the cold buffer used for polymerization containing magnesium acetate. At various times before measuring DNA synthesis at 38°C, samples of the cores were mixed with the triphosphates and incubation at 0°C was continued. For determination of the residual rate of DNA synthesis, the mixtures were transferred to 38°C, and, at 0, 15, 30, 60, 90, 120, 150, and 180 min (after transfer), trichloroacetic acid-insoluble radioactivity in 25-μl samples was determined. The concentrations of triphosphates and other components of the reaction mixtures are specified in the text. The total volume (0.25 ml) contained 1.5×10^{12} cores.

[b] Initial activity velocities are expressed as picomoles of dTMP produced per hour per reaction. The extent of the reactions after 3 h (in parentheses) is expressed as picomoles of dTMP. Column headings: (A) No further addition was made to core samples; (B) 0.02% NP-40 (final concentration) was added together with the triphosphates; (C) 0.02% NP-40 was added during transfer to 38°C.

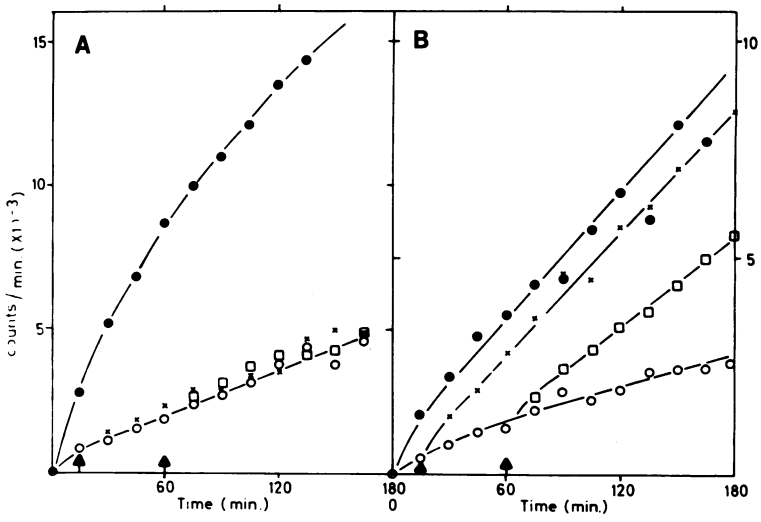

FIG. 1. *(A) Effect of NP-40 on endogenous transcription in AMV cores. Reactions were composed as described in the text. The total volume was 0.40 ml and contained 3.2 × 10^{12} core particles. At 5 min before transfer to 38°C, the cores were mixed with the triphosphates on ice. One reaction received 0.02% NP-40 simultaneously with the triphosphates (●); another reaction (○) got no NP-40; to the remaining two reactions 0.02% NP-40 was added after 15 min (×) for 60 min (□) of incubation at 38°C (arrows). The input radioactivity per point was approximately 300,000 cpm. A background of 230 cpm has been subtracted. (B) Effect of ATP on endogenous transcription in AMV cores. Reactions were carried out as described for (A), but contained 2.5 × 10^{12} cores, and 1 mM ATP was substituted for NP-40. Symbols: No ATP (○); ATP added simultaneously with the deoxyribonucleoside triphosphates (●); ATP added after 15 min (×) or 60 min (□) of incubation (arrows) at 38°C.*

TABLE 2. *Effect of 60-min preincubation at 0°C on the residual rate of DNA synthesis*[a]

Present during preincubation	Added at transfer to 38°C	Initial velocity (pmol of dTMP/h per reaction)	Extent of reaction after 3 h (pmol of dTMP)
Mg^{2+}, dNTP's[b]	No addition	0	0
Mg^{2+}, dNTP's, NP-40	No addition	84	138
Mg^{2+}, dNTP's	dNTP's	65	94
Mg^{2+}, dNTP's	dNTP's, NP-40	104	162
Mg^{2+}	dNTP's, NP-40	110	145
dNTP's	Mg^{2+}	41	61
Mg^{2+}, dNTP's, ATP	No addition	4	12
Mg^{2+}, ATP	dNTP's	77	96

[a] Total volume of the reactions was 0.30 ml; each reaction contained 1.1 × 10^{12} cores. After mixing, the reactions were left on ice (0°C) for 60 min. They were then transferred to 38°C for determination of the residual rate of DNA synthesis (as described for Table 1). The concentration of magnesium acetate (when present) was 7 mM; the concentration of NP-40 (when present) was 0.02%; and the concentration of ATP (when present) was 1 mM.

[b] dNTP's, Deoxynucleoside triphosphates.

near-linear shape of the incorporation curves in the absence of ATP and NP-40 over a period of 3 h (Fig. 1) argued against this possibility.

Table 1 shows that the low rate of DNA synthesis is caused by an event that occurs (at least in part) when cores are preincubated with the triphosphates in the cold in the absence of NP-40. After 1 h of preincubation at 0°C, virtually no DNA synthesis took place upon subsequent transfer of the reactions to 38°C. When reactions were transferred to 38°C just after mixing, the rate of DNA synthesis in the absence of NP-40 was about 50% of the rate measured in the presence of detergent (Table 1). NP-40 could prevent this event from occurring, but could not reverse the event (Table 1). (The slight reversal seen with NP-40 is only apparent; see below.)

The presence of a nucleoside triphosphatase in cores. Table 2 shows the rates of DNA synthesis at 38°C after a 60-min preincubation of cores with nucleoside triphosphates at 0°C under various conditions. No DNA synthesis occurred after this preincubation, but when new triphosphates were added just before the reaction was transferred to 38°C, the DNA synthesis proceeded as if the mixtures had not been preincubated at all. This result strongly suggested the presence of a nucleoside triphosphatase in the cores, and indeed all the results may be explained by assuming that the triphosphatase has the following properties: (i) it is more active at 0°C than at 38°C; (ii) it is virtually completely inhibited by low concentrations of NP-40; and

(iii) it is stimulated by magnesium ions.

An experiment was carried out to try to demonstrate the existence of such a phosphatase directly by following the fate of the ^{3}H-labeled dTTP during the incubation of the deoxynucleoside triphosphates with cores (Fig. 2). A phosphatase with the properties predicted was definitely present in the cores. At 0°C the enzyme degraded dTTP to dTDP (Fig. 2A), but when 0.02% NP-40 was added no degradation of dTTP could be observed (Fig. 2B). Likewise, the omission of magnesium ions from the reaction mixture inhibited the degradation of dTTP (Fig. 2C). At 38°C a very similar pattern was found for the degradation of dTTP (Fig. 2D to F). At this temperature the phosphatase was initially very active, as evidenced by the rapid initial degradation of dTTP, but the enzyme was inactivated very rapidly (Fig. 2D). Also, the initial phosphatase activity was inhibited by NP-40 (Fig. 2E). This result explains why full activity of DNA synthesis in the absence of NP-40 was not observed even when the reactions were transferred to 38°C immediately after addition of triphosphates. Also, the result explains the apparent reversal seen with NP-40 after incubation of the cores with the triphosphates at 0°C (Table 1). The two last panels (G and H) of Fig. 2 show that a similar triphosphatase activity is present in intact AMV virions. (See below for a further discussion of these two panels.)

Figure 3 shows that the residual rate of DNA synthesis after various periods of preincubation of the cores with deoxynucleoside triphosphates at 0°C correlates well with the concentration of dTTP remaining after the preincubation. In this experiment, NP-40 (0.02%) was added when the reactions were transferred to 38°C to inhibit further degradation of dTTP at this temperature (Fig. 2D and E). It is remarkable that dTTP is virtually completely converted to dTDP after 30 min at 0°C, but that no formation of dTMP

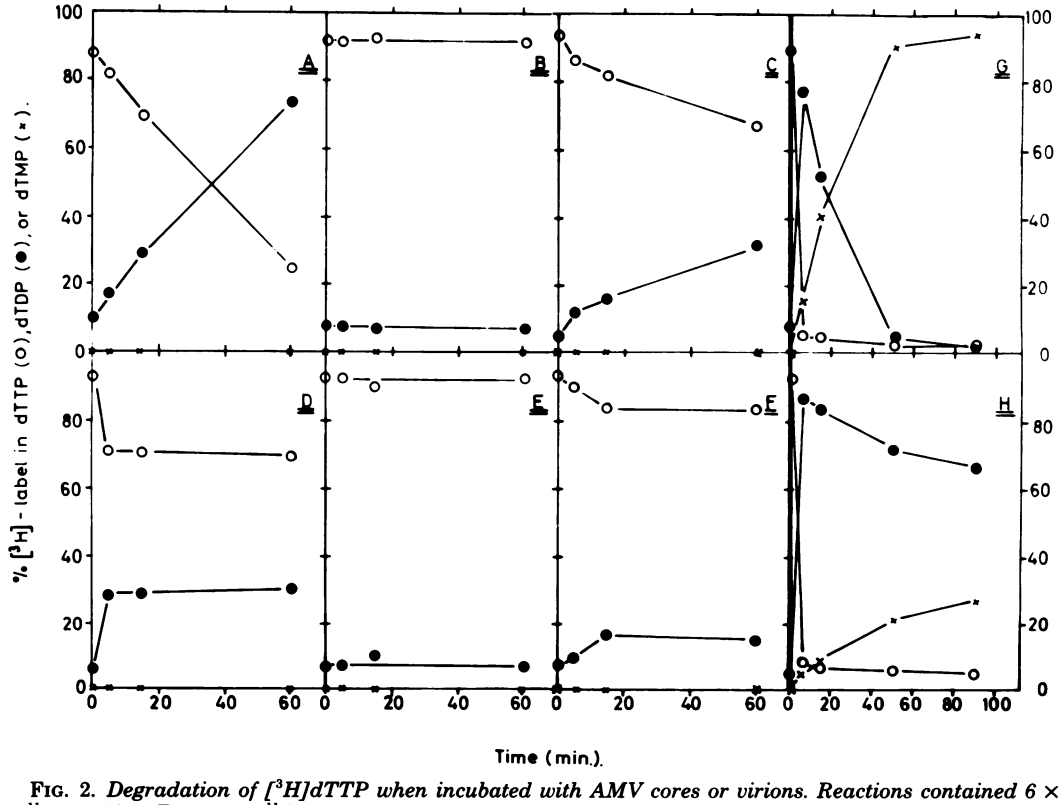

FIG. 2. *Degradation of [^{3}H]dTTP when incubated with AMV cores or virions. Reactions contained 6 × 10^{11} cores (A to F) or 2 × 10^{11} intact virions (G and H) in a total volume of 0.15 ml. The amount of radioactivity in dTTP, dTDP, and dTMP at times indicated was determined as described in the text. The total radioactivity (12,000 on Whatman paper) did not decrease measurably during the course of the experiment. (A) Cores, no NP-40, 0°C, 7 mM magnesium acetate; (B) cores, 0°C, 0.02% NP-40, 7 mM magnesium acetate; (C) cores, 0°C, no NP-40, magnesium acetate omitted; (D) cores, 38°C, no NP-40, 7 mM magnesium acetate; (E) cores, 38°C, 0.02% NP-40, 7 mM magnesium acetate; (F) cores, 38°C, no NP-40, magnesium acetate omitted; (G) virions, 0°C, no NP-40, 7 mM magnesium acetate; (H) virions, 0°C, 0.02% NP-40, 7 mM magnesium acetate.*

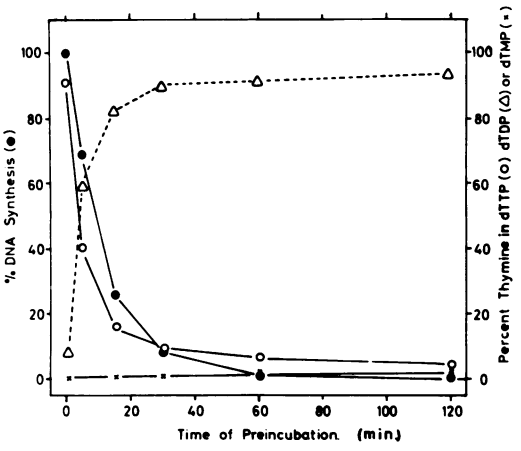

FIG. 3. *Correlation of residual rate of DNA synthesis with the residual amount of dTTP after various periods of preincubation of cores with deoxynucleoside triphosphates at 0°C. The reactions contained 2.1 × 10^{12} cores in a total volume of 0.35 ml. They were assembled and preincubated on ice for the time periods indicated; then 0.02% NP-40 was added to prevent further degradation of dTTP (Fig. 2). Samples were withdrawn for chromatographic determination of dTTP, dTDP, and dTMP. The rest of the reactions were transferred to 38°C to determine the rate of DNA synthesis (as for Fig. 1). Initial velocities are here expressed as percentage of the rate of the reaction that was not preincubated (this rate was 10,100 cpm/h per 25-μl reaction).*

takes place over the entire period of incubation (2 h, Fig. 3). Thus, the phosphatase must be rather specific for nucleoside triphosphates.

The triphosphatase is irreversibly denatured at 38°C whether nucleoside triphosphates are present or absent. This was seen from an experiment (not shown) where cores were incubated at 38°C for 30 min with nucleoside triphosphates omitted. The cores were then cooled to 0°C and incubated for another 60 min at this temperature. The rate of DNA synthesis then was determined at 38°C. This rate was the same whether the deoxynucleoside triphosphates were added at the beginning or at the end of the 60-min incubation at 0°C.

Source of the nucleoside triphosphatase. The two panels (G and H) at the right side of Fig. 2 show the fate of [^{3}H]dTTP when deoxynucleoside triphosphates (total concentration, 0.31 mM) were incubated at 0°C with a suspension of AMV virions (threefold less particles than for the corresponding assay with cores). A very rapid conversion of dTTP to dTDP took place. dTDP was subsequently converted to dTMP at a much slower rate (Fig. 2G). This latter reaction was inhibited by NP-40 (Fig. 2). The conversion of dTTP to dTDP was also detergent inhibited. Although not apparent from Fig. 2 (G and H), this was seen from an experiment using 10-fold less virions than for the experiment shown in Fig. 2 (Table 3). Very similar kinetics were observed for the degradation of (0.31 mM) α-[^{32}P]ATP, when this compound was incubated with AMV virions (not shown). Also this adenosine triphosphatase activity is detergent inhibited. Table 3 presents a comparison of the triphosphatase activities of AMV virions and cores. From the half-lives of dTTP with virions and cores (Table 3) it may be concluded that a contamination of cores with less than 0.3% of a virion triphosphatase is sufficient to explain our results with nucleotide breakdown by AMV cores.

Deoxyribonucleotide kinases in AMV cores. To explain the results presented above, nucleoside diphosphokinases must be present in the cores (Fig. 1B). Such an enzymatic activity has been reported to be associated with the AMV reverse transcriptase (4) and in cores of Rous sarcoma virus (12). An experiment was performed in the presence of 1 mM ATP (results not shown), where dGTP was sustituted by dGDP or dGMP or omitted from the transcription reaction. The incorporation of radioactivity in trichloroacetic acid-insoluble material was strictly dependent upon a source of deoxyguanosine nucleotides. Apparently, dGDP substituted very well for dGTP. The monophosphate (dGMP) substituted very poorly for dGTP.

DISCUSSION

The results show that AMV cores contain an enzymatic activity which breaks down dTTP to dTDP at 0°C. It is not known whether the three other deoxynucleoside triphosphates are broken down too, but this seems likely, since ATP is broken down.

TABLE 3. *Comparison of the phosphatase activity of AMV virions and cores*[a]

Source of phosphatase	NP-40 added	Half-life (min) at 0°C of:	
		dTTP	dTDP
Cores (6 × 10^{11} particles)	–	10-40[b]	ND[c]
	+	>300[b]	ND
Virions (2 × 10^{11} particles)	–	<0.1[d]	20
	+	0.2-0.3[d]	150

[a] When not otherwise stated, the numbers are based on the data of Fig. 2.

[b] Small variations of the triphosphatase activity were observed. The numbers given are the outer limits.

[c] ND, Not done; half-life is too long to measure.

[d] These numbers are based on an experiment where only 2 × 10^{10} virions were used per reaction. The half-life observed has been divided by 10. The observed rates of formation of dTMP justify this operation.

The triphosphatase of the cores is completely inhibited by low concentrations of NP-40. The activity is initially higher at 38°C than at 0°C, but breakdown of triphosphates soon terminates at 38°C. This inactivation occurs also in the absence of triphosphates.

The phosphatase activity of the cores appears specific for nucleoside triphosphates, since a conversion of diphosphates to monophosphates (or to nucleosides) could not be demonstrated with cores.

A similar triphosphatase is present in AMV virions. When assayed at 0°C there is at least 300-fold more of the activity in nondisrupted virions than in cores. In virions the activity is also very specific for triphosphates. The subsequent conversion of diphosphate to monophosphate occurs at a rate more than 100-fold slower. It is not known whether both conversions are due to the same enzyme. Virions break down ATP by the same kinetics as they break down dTTP. Both triphosphatase and diphosphatase activities of virions are inhibited by 0.02% NP-40. This concentration of the detergent is not sufficient to produce cores, as the vast majority of virions still band at the same density (1.16 g/ml) after treatment with this concentration of detergent (17; not shown).

The fact that the residual triphosphatase activity of cores is strongly inhibited by detergent and highly thermolabile, and the fact that the triphosphatase activity of the virions is also inhibited to some degree by low concentrations of NP-40, indicate that the activity is dependent upon a relatively intact state of the membrane. Since the cores are isolated after disruption of virions with 0.25% NP-40, probably only fragments of membranes are trapped with the cores. Thus, the triphosphatase of the cores may be in a nonphysiological state. This notion agrees with the high thermolability of the enzyme in cores. Some variation (1.26 to 1.29 g/ml) of the core density was seen in the various preparations; also some variation (two- to fourfold) of the associated triphosphatase activity was found. There is probably a correlation between these two variables, but we did not carefully investigate this, since the small variations of core density occurred in an unpredictable manner. The actual contamination of cores with membranes is probably higher than the contamination with triphosphatase (which is 0.3% or less), since such a small content of lipid would hardly be expected to create measurable variations in the core density.

All of the experiments presented here were carried out using unbanded virions for the core preparation. The virions were only pelleted from the viremic plasma by differential centrifugation. There are two reasons for this: (i) the virions banded (1.16 g/ml) in a relatively homogeneous manner after the differential centrifugations, and (ii) according to Stromberg (15) it is not good for the yield of cores to subject the virions to hypertonic solutions prior to the detergent treatment. However, some experiments (not shown) were carried out with cores made from banded virions and no difference from the results presented here were found, notably, no difference in the amount of triphosphatase.

Many activities involved in nucleotide metabolism have been reported to be present in virions of RNA tumor viruses (see ref. 17 for review), but since few of the enzymes have been purified and characterized it is not possible to tell how many of these activities are due to the combined action of several enzymes or how many are different activities of the same enzyme. The triphosphatase activity described herein seems similar to the general triphosphatase activity described by Roy and Bishop (14). These authors did not investigate deoxynucleotides as substrates, but their activity was strongly inhibited by Triton N101. The relationship to the membrane-associated adenosine triphosphatase of AMV (2) is unclear. Also this enzyme is known to be inhibited by detergent (14), but is not known to break down deoxynucleoside triphosphates (2).

The amount of the membrane-associated adenosine triphosphatase in AMV depends upon the source of the virions. Thus, virus grown in myeloblasts contains high levels of the enzyme, whereas virus grown in fibroblasts contains only little of the enzyme (18). In this study we have used virions isolated from viremic plasma (i.e., from myeloblasts). These virions can be obtained in much larger quantities than virions grown in fibroblasts.

The triphosphatase of AMV cores is greatly reduced compared to the intact AMV virion, and the remaining activity can be completely inhibited by low levels of NP-40 or by preincubating the cores at 38°C for a short time. Thus, the cores from plasma-grown AMV virions provide a suitable system for studying the DNA synthesis of AMV without complications from nucleotide breakdown during the reaction. The cores can be isolated without measurable degradation of the template RNA, and from reactions where higher concentrations of deoxynucleoside triphosphates were used, it was possible to isolate small amounts (1 to 1.5% of the total amount of DNA synthesized) of full-length DNA copies fully complementary to the 35S AMV RNA subunits (unpublished data).

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